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Cytokine regulation of adult neurogenesis: The immune system provides neuronal precursors for adult neurogenesis

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Abstract

Adult neurogenesis, or the creation of new neurons in the adult brain, is an evolutionarily conserved process that occurs in both vertebrate and invertebrate species, from the neurogenic niche of the crayfish *Procambarus clarkii* to the subventricular zone of the olfactory bulb and subgranular zone of the hippocampus in humans. The mechanisms regulating adult neurogenesis are of interest to the medical community because of the promise of new drug targets and therapies for neurological disorders. Recent findings in *P. clarkii* have shown that neuronal precursors must be added to the niche from an extrinsic source because even though the pool of neuronal precursors in the niche is never depleted, the cells themselves are not self-renewing. *In vitro* and *in vivo* findings have shown that the immune system is likely the extrinsic source. Here, three protocols are used to extend these findings and to further characterize the relationship between the immune system and adult neurogenesis in *P. clarkii*. Cells labeled with 5-bromo-2'deoxyuridine (BrdU) were quantified in the neurogenic niche and immune system tissues (the anterior proliferation center [APC] and posterior hematopoietic tissue [HPT]) at standardized time points over a 21 day period. In the first protocol, only BrdU was injected into the crayfish on day 0. In the second both BrdU and r-AST1, an immune system modulator, were injected on day 0, and in the third protocol, BrdU was injected on day 0 and r-AST1 was injected on day 1. The resulting data show that manipulating the immune system of *P. clarkii* alters the timeline by which neuronal precursors arrive in the neurogenic niche, demonstrating that neuronal precursors in the crayfish are derived from the innate immune system.

Introduction

The timeline by which new neurons are born and integrated into the brain's complex circuitry has been highly disputed over the past hundred years, with dogma originally stating that animals are born with all of the neurons they will ever have. However, evidence has emerged over the last fifty years that shows that neurons are, in fact, created and functionally incorporated into electrical pathways throughout life in many animals. The phenomenon of adult neurogenesis, the creation of new neurons in the adult brain, is now acknowledged in the hippocampus and olfactory bulb of mammals, including humans (Kuhn, Dickinson-Anson et al. 1996; Eriksson, Perfilieva et al. 1998). Further, understanding the mechanisms underlying adult neurogenesis holds promise of new treatment targets for central nervous system diseases, including brain tumors and neurodegenerative diseases such as Parkinson's and Alzheimer's.

Neurons are born in the adult brains of a range of species, including invertebrates such as crustaceans. The present study seeks to confirm and to further specify the mechanisms by which new neurons are created in the adult brain by utilizing the crayfish *Procambarus clarkii,* a reliable invertebrate model system. Adult neurogenesis in crustaceans and in mammals is highly conserved, as demonstrated by the many parallels between these processes. For example, both crustaceans and mammals have a highly vascularized neurogenic niche and a defined precursor cell lineage composed of 3-4 cellular generations that is regulated by environmental cues, serotonin and nitric oxide (Sullivan, Sandeman et al. 2007; Beltz, Zhang et al. 2011). These and many other parallels suggest that discoveries pertaining to adult neurogenesis in the crayfish model may be applicable to adult neurogenesis in mammals. The current studies examine the relationship between the immune system and adult neurogenesis, and further test the finding that the immune system provides neuronal precursors in the crayfish brain (Benton, Kery et al. 2014).

Adult neurogenesis: a controversial history

The phenomenon of adult neurogenesis in mammals was first demonstrated by Altman in the 1960s (Altman 1962). However, because adult-born neurons would have to integrate into the brain's complex circuitry, a seeming impossibility considering the necessity of stable circuits to maintain function throughout life, the birth of neurons in the adult brain was widely rejected as a general occurrence by scientists until the 1990s (Gould, Reeves et al. 1999, Nottebohm 2002). In addition, many people who suffer brain injury due to stroke, trauma or aging never recover function, and thus the clinical picture also throws doubt into the possibility of adult neurogenesis.

However, as more efficient cell labeling techniques were developed that allowed researchers to track the birth of new neurons, the process of natural adult neurogenesis in many vertebrate and invertebrate species was slowly accepted. The original labeling methods involved ^{3}H-thymidine incorporation and detection by autoradiography, which involved weeks or months of processing time (Altman 1962, Altman and Das 1965). In the 1990s, labeling with 5-bromo-2'deoxyuridine (BrdU) followed by immunocytochemistry became the most popular method for labeling new neurons in the brain (Kuhn, Dickinson-Anson et al. 1996, Eriksson, Perfilieva et al. 1998, Gould, Reeves et al. 1999). Most recently, methods utilizing viral introduction of green fluorescent protein (GFP)-vector (van Praag, Schinder et al. 2002; Carleton, Petreanu et al. 2003) and carbon-14 radiodating (Bhardwaj, Curtis et al. 2006) have been introduced.

In the first widely accepted example of adult neurogenesis, Nottebohm found newborn neurons in the high vocal center (HVC) of adult songbirds (Nottebohm 2002). Studies in rodents have since shown that chemically preventing neurogenesis in mice caused deterioration of the olfactory bulb, indicating that a constant addition of cells is required for the bulb's integrity (Imayoshi, Sakamoto et al. 2009). In addition, Imayoshi showed that over time the number of

cells in the hippocampus increases, indicating that new cells are incorporated into the region throughout life. To prove the functionality of new neurons in the hippocampus, van Praag et al. (2002) used GFP-labeling methods to show that neurons born in adulthood are, in fact, electrically functional in the hippocampus, indicating that the new neurons are successfully integrated into the brain's existing circuits (van Praag, Schinder et al. 2002). Further, Gould et al. (1999) demonstrated adult neurogenesis in the subventricular zone (SVZ) of macaque monkeys and Carleton et al. (2003) demonstrated functional incorporation of neurons generated in the SVZ in the olfactory bulb of mice (Gould, Reeves et al. 1999; Carleton, Petreanu et al. 2003) These authors, among others, collectively proved the existence of neuronal precursors underlying adult neurogenesis in the subgranular and subventricular zones in a variety of model systems, and have shown that these areas contribute new neurons to the hippocampus and olfactory bulb, respectively (Eriksson, Perfilieva et al. 1998; van Praag, Schinder et al. 2002; Imayoshi, Sakamoto et al. 2009).

BrdU labeling to track adult neurogenesis

BrdU labeling is one of the most effective methods by which to label newborn cells (used efficiently in Gould, Reeves et al. (1999) and Benton et al. (2014)) and is the method used in the present study. BrdU, a thymidine analog, is incorporated into the DNA of any cell that goes through S phase prior to the clearing time, which is the time it takes for the amount of BrdU in a system to dissipate enough such that the label incorporation no longer reaches detectable levels. BrdU is relatively easy to detect using immunohistochemical techniques. However, one potential issue with the BrdU labeling method is that if a cell containing BrdU divides many times,

diluting the label with each division, the label will eventually no longer be visible via immunohistochemistry in long cell lineages.

Crayfish as a non-vertebrate model system for adult neurogenesis

The crayfish is a relatively simple, effective model system for investigating adult neurogenesis. Crayfish possess bilateral neurogenic niches which are located on the ventral surface of the brain. Each end of the niche tapers into a migratory stream that leads to either the lateral proliferation zone (LPZ) of Cluster 10 or the medial proliferation zone (MPZ) of Cluster 9 (Figure 1). Cells produced by the division of 1st-generation neuronal precursors (functionally analogous to neural stem cells in mammals) in the niche traverse these migratory streams to cell clusters in the brain, where they will divide again and their descendants will differentiate into neurons.

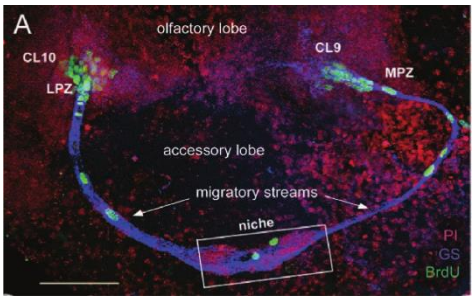


Figure 1: Confocal microscopical image of the ventral side of the crayfish brain. This image shows the niche, migratory streams, medial and lateral proliferation zones and Clusters 9 and 10. Figure adapted from Benton, Zhang et al. (2011)

Previous experiments have shown that crustaceans undergo neurogenesis throughout their entire life span. Harzsch et al. (1999) demonstrated that *Homarus americanus* (lobster) undergoes life-long neurogenesis in the olfactory pathway and Sullivan and Beltz (2005) showed that the phenomenon occurs in Cluster 10 (containing olfractory interneurons) and in the visual system in the crayfish *Cherax destructor* (Sullivan and Beltz 2005). Thus, like mammals, in which the presence of adult neurogenesis has been confirmed in the olfactory bulb and hippocampus, the process has also been confirmed in a few, distinct locations in crustaceans: the

olfactory lobe (Harzsch, Miller et al. 1999), which is analogous to the mammalian olfactory bulb, and the accessory lobe (Sullivan and Beltz 2005), a center for higher order processing similar to the hippocampus, and the visual system (Chaga, Lignell et al. 1995; Sullivan and Beltz 2005; Benton, Chaves da Silva et al. 2013).

The crayfish equivalent of mammalian neuronal stem cells, termed 1st-generation neuronal precursors, are located in the neurogenic niche (Sullivan, Sandeman et al. 2007). Unlike in mammals, BrdU labeling experiments show that when these 1st-generation cells divide, *both* of the daughter cells migrate from the niche through the migratory streams and into the LPZ or MPZ associated with interneuron clusters 10 and 9, respectively (Figure 2) (Zhang, Allodi et al. 2009). In the clusters, the cells divide again and begin to differentiate into neurons (Sullivan and Beltz 2005). Once they have differentiated, local interneurons with their somata in Cluster 9 project to both the olfactory and accessory lobes, while projection interneurons in Cluster 10 project to either the olfactory or accessory lobe (Sullivan and Beltz 2005).

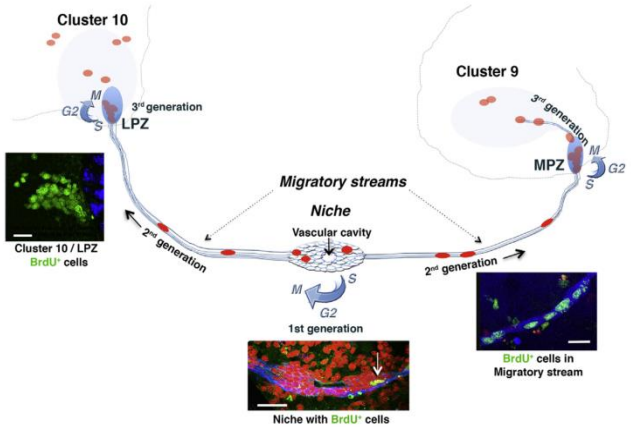


Figure 2: Schematic showing the neuronal precursor lineage. Neuronal precursors divide in the neurogenic niche, migrate and divide in the streams and arrive in the LPZ of Cluster 10 and the MPZ of Cluster 9, where they differentiate into neurons and project either to the olfactory and/or accessory lobe. Figure adapted from (Benton, Kery et al. 2014).

Typically, stem cells are thought of as long-lived and self-renewing. The NIH defines a stem cell as "cells with the ability to divide for indefinite periods in culture and to give rise to

specialized cells" (http://stemcells.nih.gov/info/basics/Pages/Default.aspx). However, as stated above, BrdU labeling experiments show that both daughter cells of the 1st-generation neuronal precursors migrate away from the niche and towards the clusters (Benton, Chaves da Silva et al. 2013). This would imply that since the niche contains only a few hundred cells, the neurogenic capacity of the crayfish would be quickly depleted. On the contrary, 1st-generation neuronal precursors are observed throughout the twenty-year life span of the crayfish, and the pool of precursors is never depleted (Benton, Chaves da Silva et al. 2013). Thus, if neuronal precursors in the crayfish niche are not self-renewing, yet are never depleted, the pool must be replenished by a source extrinsic to the niche (Beltz, Zhang et al. 2011).

The immune system as the likely source of neuronal precursors

The immune system is a logical source of neuronal precursors because the niche is an isolated structure that is surrounded by blood vessels, providing direct access for hemocytes (e.g. blood cells) to the niche. Multiple lines of evidence have recently emerged to support the immune system as the source of neuronal precursors in crayfish. In a first set of *in vitro* experiments by Benton et al. (2011), the attraction of cells from various organs of the crayfish *Procambarus clarkii* to the neurogenic niche was investigated. Hemocytes, as well as cells from the hepatopancreas, hematopoietic tissue, and green gland were labeled with CellTracker Green and co-cultured with whole brains that were desheathed to expose the niches. Of these cell types, only hemocytes were consistently attracted to the niche, indicating that cells from the immune system possess an intrinsic affinity for the neurogenic niche.

In addition, the number of cells in the niche is influenced by the total number of circulating hemocytes, indicating a strong relationship between the immune and nervous systems. Benton et

al. (2014) found that by ablating half of the HPT, the total hemocyte count decreased, as did the numbers of cells in the niche. However, it was also found that a recombinant-astakine 1 injection (described below) rescued both the numbers of circulating cells and the number of cells in the niche.

Further, *in vivo* experiments by Benton et al. (2014) labeled hemocytes in "donor" crayfish with 5-ethynyl-2'-deoyuridine (EdU), a thymidine analog that is functionally similar to BrdU. Blood taken from donor *P. clarkii* and injected into an unlabeled recipient resulted in EdU-labeled cells in the niche, streams, and clusters on successive days. These cells eventually label for orcokinin in Cluster 9 and SIFamide in Cluster 10, the transmitters typically expressed by neurons in these areas (Benton, Kery et al. 2014). These data demonstrate that hemocytes have the capacity to generate cells with neuronal properties in the adult brain.

The crayfish immune system

Unlike mammals, crayfish possess only an innate immune system (Soderhall, Kim et al. 2005). The fact that crayfish lack an adaptive immune system is potentially beneficial to our research as it decreases the likelihood of crayfish rejecting a blood transfusion. The crayfish hemolymph is composed of three cellular classes, the relatively undifferentiated phagocytic hyaline cells, phagocytic semi-granular cells and granular cells, involved in fighting off microbes (Soderhall 2013). The precursors for hyaline, semi-granular, and granular cells are produced by immune tissue through a process called hematopoiesis. The immune system tissue is composed of two regions, the posterior hematopoietic tissue (HPT) and the anterior proliferation center (APC) (Figure 3) (Chaga, Lignell et al. 1995; Soderhall 2013). These tissues are located on the

dorsal surface of the stomach and have direct access to the brain through the dorsal artery (Figure 3).

The HPT is a highly proliferative region and consists of approximately 1.4E6 cells adhered both to an extracellular matrix and in tear-drop shaped aggregates termed lobules (Chaga, Lignell et al. 1995; Soderhall, Bangyeekhun et al. 2003). Five types of cells are known to mature in the HPT in preparation for release into the hemolymph. Type 1 cells are relatively undifferentiated, type 2 cells are precursors to both semi-granular and granular cells, types 3 and 4 are precursors to granular cells and type 5 cells are precursors to the semi-granular cells (Chaga, Lignell et al. 1995; Soderhall 2013).

The APC is a region of the immune system located in the cor frontale (auxiliary heart), that is highly vascularized and has direct access to the brain through the dorsal median artery (Figure 3) (Noonin, Lin et al. 2012, Chaves da Silva, Benton et al. 2013). Like the HPT, cells in the APC are highly proliferative (Noonin et al. [2012] found even more proliferation in the APC than HPT), but

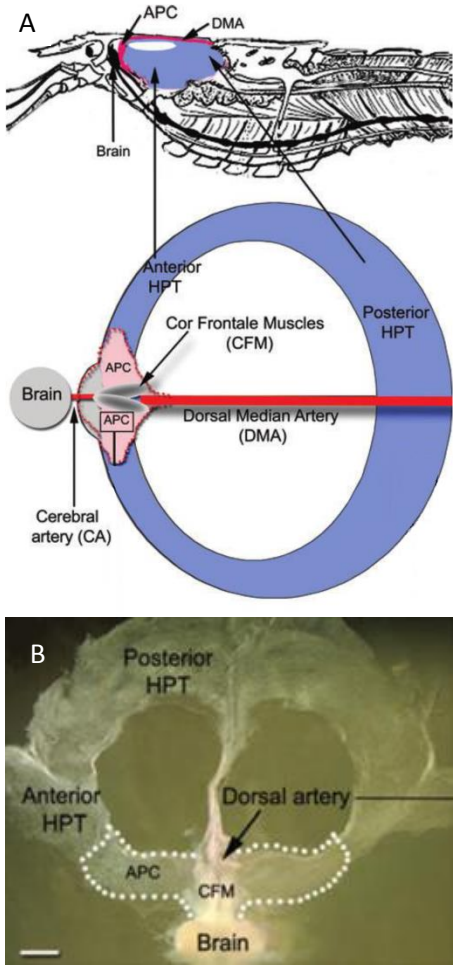


Figure 3: Schematic of the crayfish immune system. A: diagram of the immune tissue sitting on top of the crayfish stomach (DMA= dorsal median artery). B: Image of dissected immune system tissue showing the APC, HPT, brain, and DMA. Figure adapted from (Chaves da Silva, Benton et al. 2013).

unlike the HPT, many of these cells are distributed into rosettes, structures thought to be related to stem cells whose progeny will have an ectodermal fate (Chaves da Silva, Benton et al. 2013).

Regulation of the crayfish immune system:

The ability to regulate the release and production of blood and immune cells is vital to fight off infection and for general, every-day survival of an organism. Soderhall (2013) describes astakine 1, a cytokine and a prokineticin (mammalian) homologue that is involved in regulating the differentiation and release of cells from immune tissue into the circulation (Lin, Novotny et al. 2010, Noonin, Lin et al. 2012, Soderhall 2013). Prokineticins are a family of proteins involved in, among other things, the regulation of the immune system, neural development, circadian cycles and angiogenesis (Zhou 2006; Zhou and Meidan 2008; Watthanasurorot, Soderhall et al. 2011). Cytokines are signaling proteins released by the immune system that regulate an immune response (Cannon 2000). Astakine 1 is a cytosine-rich protein first discovered for its role in increasing immune cell proliferation in the crayfish *Pacifastacus leniusculus*. This molecule also prevents apoptosis of cells in the immune tissue by controlling levels of crustacean hematopoietic factor (CHF) and promotes the differentiation of immature cells into semi-granular cells (Noonin, Lin et al. 2012, Soderhall 2013). Endogenously, astakine 1 is found in HPT cells, hemocytes, specifically semi-granular cells, and nerve tissue (Soderhall, Kim et al. 2005; Lin, Novotny et al. 2010). For experimental purposes, astakine 1 has been sequenced and a recombinant astakine-1 (r-AST1) has been produced by the Soderhall lab (Lin, Novotny et al. 2010, Soderhall 2013).

Experiments have shown that astakine 1 is upregulated in the crayfish after injury or microbe invasion, indicating that the cytokine plays an important role in regulating an immune

response and in keeping the numbers of circulating hemocytes balanced. In addition, an injection of r-AST1 increased the total hemocyte count of crayfish, while RNA interference studies showed that silencing astakine 1 resulted in reduced numbers of hemocytes and eventually in the death of the crayfish (Soderhall 2013). Further, unpublished data (Soderhall Lab) indicates a connection between astakine 1 and serotonin regulation in the crayfish *Pacifasticus lenisculus.*

Astakine 1 controls immune cell release in the crayfish by regulating transglutaminase, a protein in the HPT that essentially glues immune cells to the HPT's extracellular matrix by binding with collagens. Transglutaminase is also known to prevent the maturation of HPT cells. Astakine 1 prevents the action of transglutaminase and thereby promotes the maturation and release of hemocytes by the HPT into the circulation (Watthanasurorot, Soderhall et al. 2011). More specifically, astakine 1 causes the maturation of cells by inducing the secretion of *Pl*Runt, a molecule characteristic of semi-granular and granular cells (Watthanasurorot, Soderhall et al. 2011). Recent experiments in *P. clarkii* have shown that at room temperature, r-AST1 causes a release of cells from the immune system and into the circulating hemolymph 12 hours post-injection (Benton, Kery et al. 2014).

Current Experiments:

As described above, neuronal precursors in the crayfish niche are not self-renewing, yet are also never depleted, indicating that they arrive in the brain from a source extrinsic to the niche. The studies described here further tested the finding that the immune system replenishes the supply of neuronal precursors in the neurogenic niche in crayfish. Thus, the first of the three experiments described in this paper is predicated on the fact that if, in fact, the neuronal precursors are arriving in the niche from an outside source, then after a single pulse of BrdU,

labeled cells should be present in the niche immediately following the pulse and then again at a later time point once the cells that are labeled in the source tissue are released and have migrated to the niche. This experiment, using a single pulse of BrdU, also examined BrdU-labeling patterns in the HPT and APC at various time points for three weeks following the injection to determine if there is a release of labeled cells from these tissues that corresponds to the arrival of BrdU-labeled cells in the niche. Additionally, a single pulse of 5-Ethynyl-2'-deoxyuridine (EdU) was used to determine the pattern of hemocyte labeling over the three weeks. EdU and BrdU have the same functionality, but EdU is visualized using a chemical method instead of the immunohistochemical method used to visualize BrdU. Thus, since hemocytes are known to have a high affinity for secondary antibodies, EdU is more reliable than BrdU to count hemocytes.

In two additional experiments, r-AST1 was used to definitively test whether promoting the release of cells from the immune system affects the timeline by which neuronal precursors re-appear in the neurogenic niche of the crayfish *P. clarkii.* Indeed, these experiments demonstrate that the arrival time of BrdU-labeled precursors in the niche can be manipulated by altering the timing of r-AST1 injection into crayfish, providing direct evidence that the immune and nervous systems are linked and strongly suggesting that the released immune system cells become neuronal precursors.

Using the same BrdU labeling technique and dissection timeline protocol as in the first experiment, r-AST1 was injected either in conjunction with or 24 hours following BrdU injection. The timeline by which BrdU-labeled (newborn) cells are present in the niche and the fraction of cells in the APC and HPT that are BrdU-labeled were determined and compared between the BrdU only experiment and the two experiments with varying times of r AST1 vs BrdU injection. Thus, the effect of astakine 1, which specifically releases cells from the immune

system, on both the numbers of BrdU-labeled cells in tissues of the immune system and in the niche were determined in order to evaluate the relationship between the release of hemocytes from immune tissues and the appearance of neuronal precursor cells in the niche.

It is also of current interest in our lab to determine the pathway by which cells travel as they are born and released from the immune tissues. Possible hypotheses include that cells migrate from the APC to the HPT where they mature in the lobules before being released into the hemolymph, or that both the APC and HPT directly release cells into the hemolymph. In addition, it is possible that cells enter the niche either from the circulation or through the dorsal median artery that connects the APC to the brain. Thus, the experiments described here aim to investigate the progression by which cells exit the immune tissues and enter the brain.

Materials and Methods

Animals

Procambarus clarkii, a species of freshwater crayfish, were used for all experiments and were housed in the Wellesley College Animal Care Facility. Throughout the experiments, the animals were kept in freshwater aquariums on a 12/12 light/dark cycle with artificial plants, and PVC tubing and ceramic mugs for shelter. During the first set of experiments, all animals were fed on Tuesday and Friday mornings and during the second and third sets of experiments they were fed more frequently (Monday, Wednesday and Friday), in order to decrease cannibalism. All animals had a carapace length of approximately 30-35mm. Both male and female animals were used in all experiments, although these were not scored independently, and pregnant females were removed from the cohort.

Injection protocols

Experimental series 1: BrdU-only experiment

P. clarkii were injected in the morning of day 0 with 100μL of BrdU (5mg/mL in saline). In addition, Jingjing Li also injected *P. clarkii* with 100 μL of 0.1mg/mL EdU.

Experimental series 2: Simultaneous BrdU and r-AST1 injection paradigm

An *Escherichia coli* system was used to synthesize recombinant-astakine 1 (r-AST1) in the lab of Irene Soderhall as described in Lin, Novotny et al. 2010, and the r-AST1 product was shipped to the Beltz Lab. *P. clarkii* were injected at 0930 on day 0 with 70 μl of 2.8E-3mg/mL r-AST1 (a concentration used in and determined for Benton et al. (2014)), and 100μL of 5mg/mL BrdU administered through the same syringe so that the animal would only receive one injection, as in the previous experiment.

Experimental series 3: 24-hour delay between BrdU and r-AST1 injection paradigm

P. clarkii were injected with 100μL of 5mg/mL BrdU at 1000 on day zero and returned to the animal care facility overnight to ensure consistent light/dark cycles. At 1000 on day one, after the removal of 5 animals for day 1 dissections, the remaining animals were injected with 70μL 3.08E-3mg/mL r-AST1.

Dissection protocols

Brains with attached niches and immune system tissues (APC and HPT) were dissected and fixed overnight at 4°C in 4% paraformaldehyde in 0.1M phosphate buffer [20mM NaH_2PO_4, 80mM Na_2HPO_4; pH 7.4 (Benton et al. 2014)]. Brains in all three experiments were dissected on days 1-8, 10, 14 and 21. In the BrdU-only experiment APCs were dissected on days 1-5, 8, 10, 14 and 21 and HPTs on days 1-8, 10, 14 and 20. In the simultaneous injection protocol APCs were dissected on days 1-8, 14, and 21 and HPTs on days 1-8, 10, 14, and 21. In the r-AST1 delay experiments APCs were dissected on days 1-2, 4-8, 10, 14 and 21 and HPTs were dissected on days 1-8, 10 and 14.

Immunohistochemical labeling

Brains, APC, and HPT were removed from fix, rinsed for 1.5 hours in 0.3% Triton in 0.1M phosphate buffer (PBTx), soaked in 2N HCl for 30 minutes, and rinsed again in PBTx for 1.5 hours. Brains were then incubated overnight at 4°C in rat anti-BrdU (1:50; Accurate Chemical OBT0030G) and mouse anti-glutamine synthetase (GS; 1:100 BD Biosciences: 610517) primary antibodies. APC and HPT were incubated overnight at 4°C in a mouse anti-BrdU antibody conjugated with AlexaFlour 488 (1:20) (Life Technologies: B35130). All tissues were then rinsed again in PBTx for 1.5 hours. Brains were then incubated overnight at 4°C in goat anti-rat CY2/488 (1:100 Jackson Immuno Research) and goat anti-mouse CY5/649 (1:100

Jackson Immuno Research) secondary antibodies and then rinsed for 1.5 hours in PBTx. All tissues were incubated for 10 minutes in propidium iodide (PI; 1:100 in PBTx), rinsed again for 1.5 hours in PBTx and then mounted using FlouroGel with tris buffer (17985-11) mounting medium and allowed to set for two days at room temperature prior to storage at 4°C. Brains were mounted with their ventral side towards the coverslip.

Data Collection

All tissue preparations were analyzed using a Leica TCS SP5 confocal microscope. An image was taken bilaterally at points A and B (Figure 4) in each APC and an image was taken bilaterally at the posterior region of each HPT, adjacent to the dorsal sinus (Figure 4). These regions were chosen due to their known proliferative nature and for the presence of landmarks in the tissue (the muscles of the APC and dorsal sinus in the HPT) that allow approximate standardization of the location of these sampling sites. Each image was taken at 40X magnification, magnified an additional 3.00 times. A 4 μm stack of 1.0 μm thick images was taken at each location and rendered as a maximum projection in 2D for analysis.

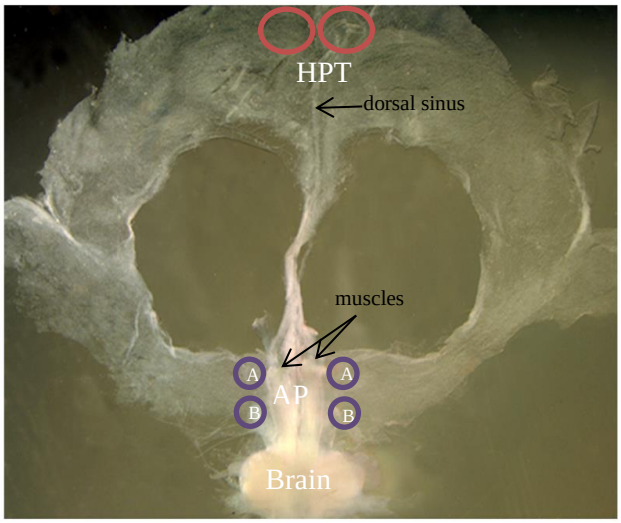


Figure 4: Schematic of dissected tissue regions and labeling sites. In the APC, 4 images were taken per animal, bilaterally at region A and region B adjacent to the muscles (shown in purple). In the HPT, 2 images were taken per animal (location circled in red) at the posterior end of the HPT, adjacent to the dorsal sinus. Images in all locations were taken at 40X magnification with an additional 3.00 times zoom. Each image was 4 μm thick. Image courtesy of Jeannie Benton.

Data analysis

Determining the number of BrdU-labeled cells in a niche

A cell in the niche was considered to be BrdU-labeled if it fluoresced at 488 nm, was labeled with PI, and was surrounded by GS immunoreactivity, which is found in the cytoplasm of all niche cells (Figure 5). This was determined by examination with the Leica software on the confocal microscope. The percent chance of finding a BrdU-labeled cell in the niche was determined by dividing the number of niches that possessed any number of BrdU-labeled cells by the total number of niches.

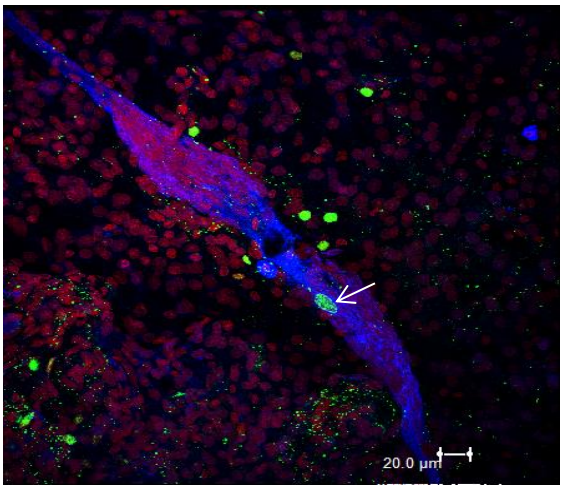
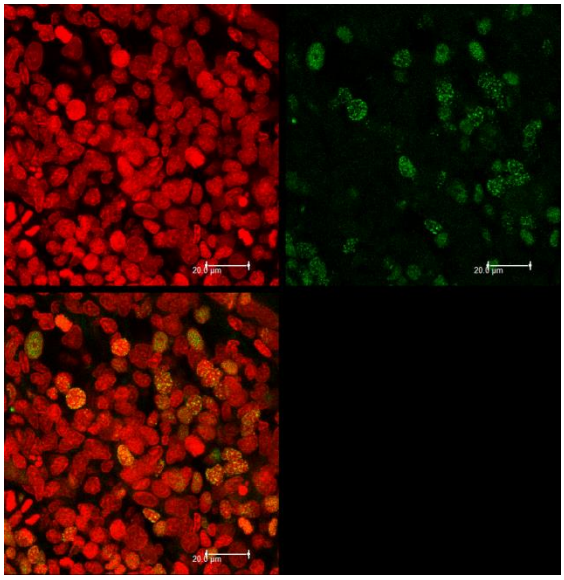


Figure 5: Representative image of a niche with a BrdU-labeled cell. The niche is outlined in blue (GS), PI is red, and the labeled cell is denoted by green labeling and a white arrow.

Determining BrdU-labeling in the APC and HPT

After all imaging was complete, the maximized, 2D stacked images of each APC and HPT region were transferred to a laboratory computer and the number of PI-labeled cells and the number of BrdU-labeled cells were counted independently (Figure 6). To make these counts, a transparency was taped to the computer screen and each cell was circled by hand and counted. In order to obtain a percentage of BrdU-labeled cells for each tissue region, the number of BrdU-labeled cells was divided by the total number of PI-labeled cells for each image. For analysis, the four regions sampled in each APC in one animal were averaged and counted as one "n"; likewise, and the two regions sampled from the HPT in one animal were averaged and each animal was counted as a single "n". It should be noted that, due to methodological constrains, at times not all four regions of each APC and both regions of the HPT were available for counting.



Thus, some averages represent fewer than the maximum number of counts. However, given the relatively small variability in these counts, these samples can still be confidently included in the data set.

Figure 6: Representative 2D, stacked 4 µm thick APC/HPT image. Top left: PI-labeled cells. Top right: BrdU-labeled cells. Bottom left: overlay of BrdU-labeled and PI-labeled cells. (40X, zoom 3.00).

Determining EdU-labeling in Hemocytes

Hemocyte samples were taken on days 1, 3, 5, 10, and 17 following injection. The Click-iT Alexa Flour 488 Imaging Kit was used to visualize EdU in hemocytes drawn from animals and placed on a slide such that they were one layer thick. 3 sample regions on each slide were visualized using confocal microscopy at 40X, zoom 3.00, and the percentage of cells labeled with EdU was determined.

Statistical analysis

A One-Way ANOVA was performed using JMP software to determine whether any differences existed in the percent of BrdU-labeled cells on day 1 in the APC across all three experimental protocols and in the HPT across all three experimental protocols. Data are presented as means +/-SD.

Results

In order to determine the timeline by which BrdU-labeled cells are found in the niche, a cohort of *P. clarkii* were injected with 5mg/mL BrdU and their brains were taken on days 1-8, 10, 14, or 21 post-injection. As expected, due to a niche cell cycle time of approximately two days and a BrdU clearing time of approximately two days, immunohistochemical labeling revealed BrdU-labeled cells in the niche on days 1-4 post-injection (peak A), but no BrdU-labeled cells on days 5-7 post-injection (Figure 7A). However, as shown in Figure 7A, BrdU-labeled cells were again present in the niche on days 8-14 (peak B) following BrdU-injection.

By day 5 post-injection, due to BrdU clearing and the cell cycle times, the cells that were labeled in the niche would have divided and migrated into the streams; hence there is a gap in labeling between days 5 and 7. This lack of BrdU labeling confirms that BrdU is not retained in the niche cells, and also means that the BrdU-labeled cells that appear in the niche on days 8-14 must have been labeled in a source tissue extrinsic to the niche. The source tissue would have been labeled at the time of BrdU injection, and the labeled neuronal precursor cells released from that tissue would then have migrated to the niche.

Given the evidence pointing to the innate immune system as a source of neuronal precursors in the crayfish, it was hypothesized that the timing of peak B could be manipulated with astakine 1, an immune system modulator known to cause the release of immune system cells 12 hours after injection (Benton, Kery et al. 2014). Thus, the labeling experiment was repeated, this time with a simultaneous injection of 5mg/mL BrdU and r-AST1 on day 0, in order to test whether the timing of appearance of BrdU-labeled cells seen in the niche on days 8-14 (peak B) was altered. We hypothesized that, if the immune system is indeed the source of neuronal precursors, the addition of r-AST1 on day 0 would accelerate the appearance of cells associated with peak B,

due to the release of cells from the immune system tissue 12 hours after r-AST1 injection (Benton, Kery et al. 2014). In other words, the injection of r-AST1 was predicted to close the "gap" between peaks A and B (i.e., the BrdU-labeled cells in the niche on days 1-4 following BrdU injection [presumably labeled as they were going through S phase in the niche], and the cells labeled in the source tissue that take time to be released into circulation and migrate into the niche on days 8-14).

As shown in Figure 7B, following a simultaneous injection of BrdU and r-AST1 on day 0, BrdU labeled cells are present in the niche at all sampled time points (there is no longer a gap period), with peaks on days 2, 4, 7 and 10 post-injection (peaks C-F in Figure 7B).

To further confirm that manipulation of the immune system via introduction of r-AST1 caused the change in the timing by which BrdU-labeled cells are seen in the niche, the timeline labeling experiment was again repeated, this time with an injection of 5mg/mL BrdU on day 0 and an injection of r-AST1 on day 1. It was hypothesized that if peak E in Figure 7B is due to the release of BrdU-labeled cells from the immune system 12 hours after r-AST1 injection, injecting r-AST1 24 hours later would cause peak E to shift 24 hours to the right, thus definitively showing that the timeline by which BrdU-labeled cells appear in the niche can be controlled by manipulating the differentiation and release of cells from the immune system.

As shown in Figure 7C, following BrdU injection on day 0 and r-AST1 injection 24 hours later, BrdU-labeled cells are present in the niche on all sampling days with peak maxima on days 1, 4 and 14 post-injection (labeled in Figure 7C as peaks G-I).

Figure 7D shows a comparison of the percent chance of finding BrdU-labeled cells in the niche across all three experimental protocols. Peak E after simultaneous injection of BrdU and r-AST1 fills the gap between peaks A and B from the BrdU only experiment, as predicted if peak

E is due to the release of immune system cells. A comparable peak 24 hours later, as hypothesized, is not identifiable when r-AST1 was injected 24 hours after BrdU. However, peak I is wider (spanning 14 days) and 43.33% taller than peak B, indicating an increase in BrdU-labeled cells and a possible delay in cells corresponding to peak E such that these combined with peak B to form peak I.

Finally, as seen on the right Y-axes of Figure 7A-C, the average number of BrdU-labeled cells found in a niche at each time point roughly follows the pattern of the percent chance of finding BrdU-labeled cells in the niche, rising and falling with the peaks.

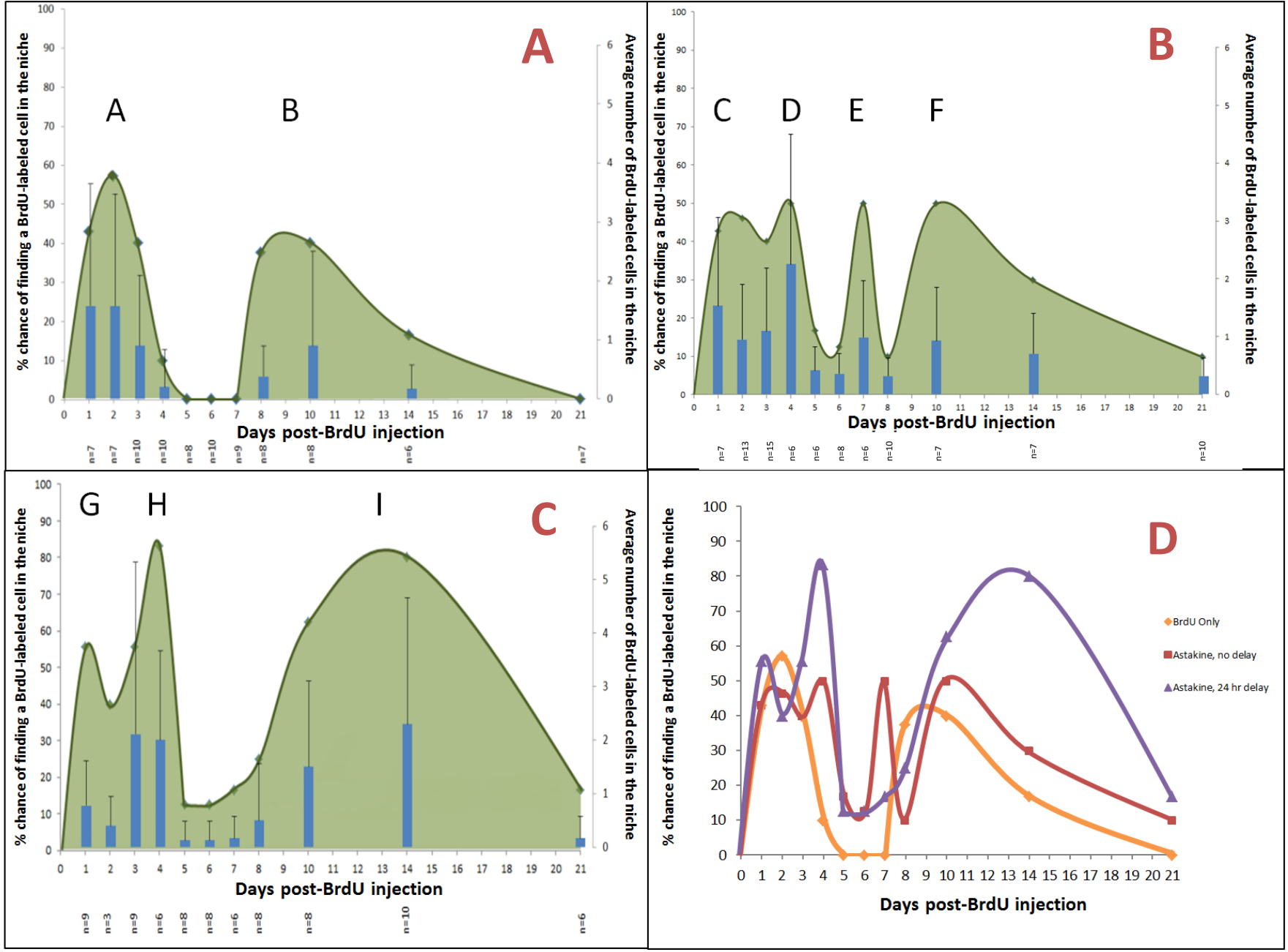


Figure 7: Injection of r-AST1, an immune system modulator, alters the timeline by which BrdU-labeled cells (neuronal precursors) are seen in the niche. *P. clarkii* were injected with (A) BrdU on day 0, (B) BrdU and r-AST1 on day 0, or (C) BrdU on day 0 and r-AST1 on day 1. For each experimental protocol brains were dissected on days 1-8, 10, 14 and 21 and the percent chance of finding BrdU-labeled cells in the niche was calculated (shown in green). In addition, the average number of BrdU-labeled cells in the niche was calculated for each time point and is represented in the blue bar graphs (mean +/- SD). Peaks in labeling are denoted by a letter above the peak and are found in (A) on days 2 and 10, in (B) on days 2,4,7, and 10, and in (C) on days 1, 4 and 14 . D: Comparison of the % chance of finding BrdU-labeled cells in the niche across all three experimental protocols.

In order to characterize the activity of immune system tissues and their response to r-AST1 injection, the percent of cells in the APC and HPT that were labeled with BrdU at days 1-8, 10, 14 and 21 in each of the three experimental protocols was determined. As shown in Figure 8, the percent of cells labeled with BrdU in the APC decreases exponentially with time in the BrdU-only protocol. In contrast, the percent of cells labeled in the HPT remains constant over the first three days after BrdU injection, then quickly drops off. The decrease of BrdU-labeled cells in the immune system tissues is followed by an increase of BrdU-labeled cells in the circulating hemolymph (Figure 8), and then by the subsequent appearance of BrdU-labeled cells in the niche (e.g., peak B [Figure 7]).

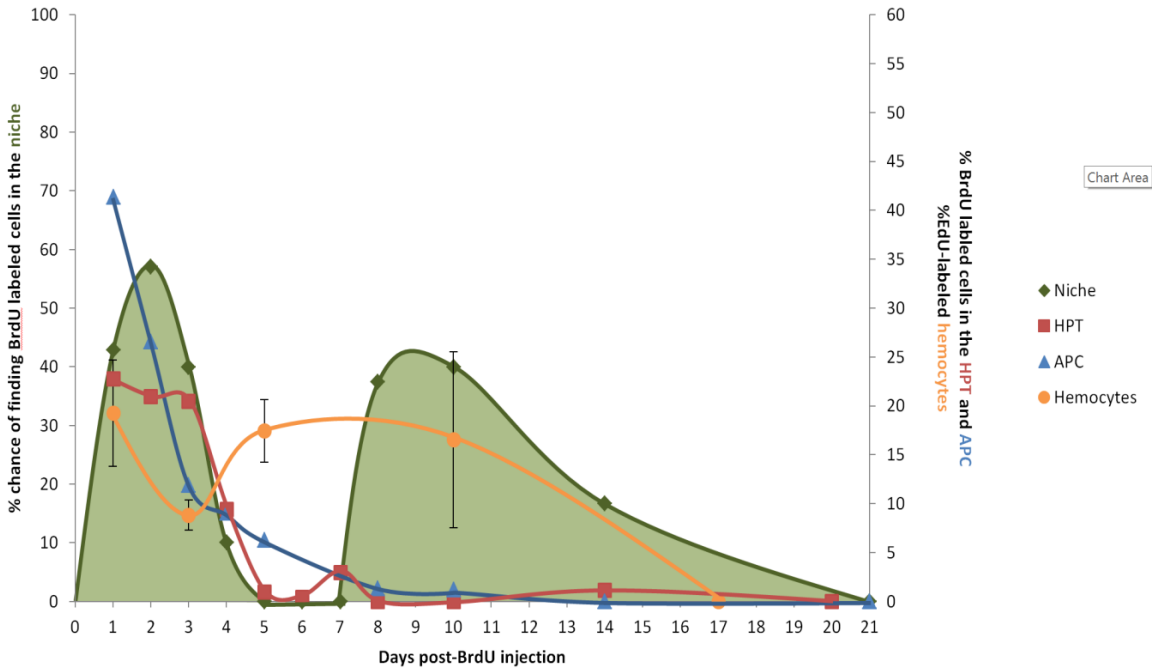


Figure 8: The percent of BrdU-labeled cells in the immune tissues (APC and HPT) decreases prior to an increase of EdU-labeled cells in the circulating hemolymph and the subsequent reappearance of BrdU-labeled cells in the niche. After a single injection of BrdU on day 0, brains, APC and HPT were dissected on days 1-8, 10, 14 and 21 post injection. The % chance of finding BrdU labeled cells in the niche as well as the % of cells that were BrdU-labeled in the HPT and APC was determined for each time point. BrdU labeling decreases exponentially in the APC but remains constant for days 1-3 in the HPT before severely dropping on day 4. BrdU-labeled cells are found in the niche days 1-4 and again days 8-14 post-BrdU injection. In addition, following an EdU injection, the numbers of circulating EdU-labeled hemocytes on days 1, 3, 5, 10 and 17 was determined: day (d)1n=3, d3n=4, d5n=4, d10n=2, d17n=unknown. The hemocyte work was done by Jingjing Li.

In the simultaneous injection of BrdU and r-AST1 protocol, the percent of cells labeled with BrdU in the APC remains relatively constant between days 1 and 2 and then drastically drops to near negligible amounts on subsequent days (Figure 9). Similarly, the percent of cells labeled with BrdU in the HPT remains level on days 1 and 2 and then drops sharply on day 3 to near negligible amounts on subsequent days (Figure 9).

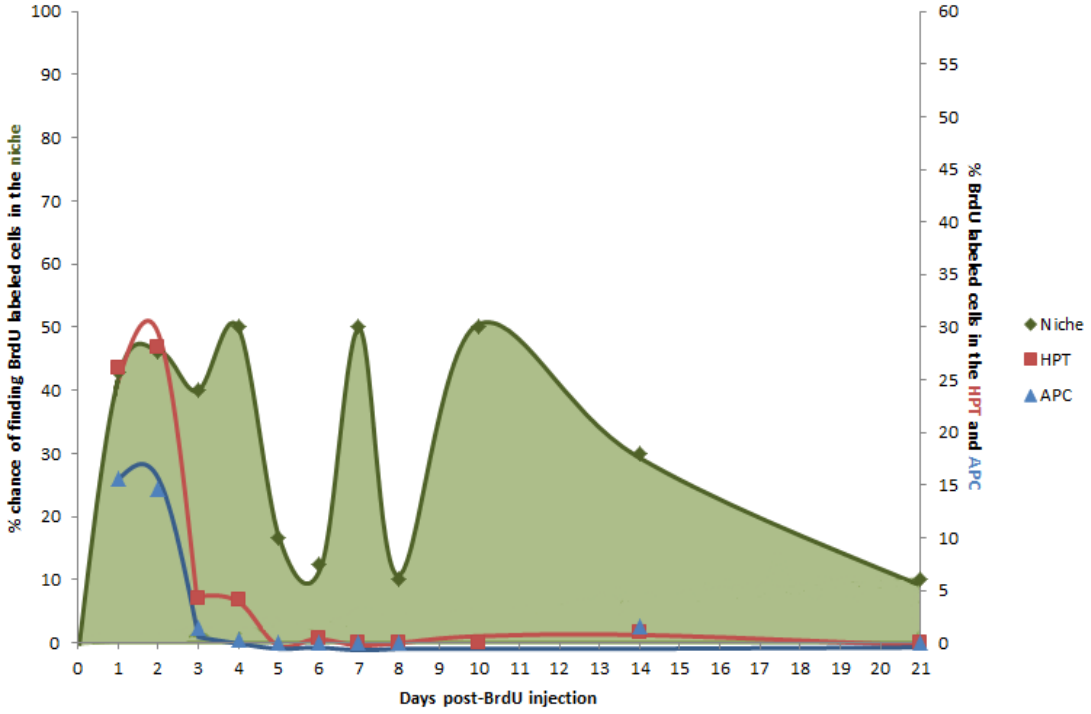


Figure 9: The percent of BrdU-labeled cells in the immune tissues (APC and HPT) remains constant on days 1 and 2 and then decreases sharply prior to the second, third and fourth labeling peaks in the niche. After a simultaneous injection of BrdU and r-AST1 on day 0, brains, APC and HPT were dissected on days 1-8, 10, 14 and 21 post-injection. The % chance of finding BrdU labeled cells in the niche as well as the % of cells that were BrdU-labeled in the HPT and APC was determined for each time point. BrdU-labeled cells are found in the niche on all days with peaks on days 2, 4, 7, and 11.

In the third protocol, with a 24 hour delay between BrdU and r-Ast 1 injection, the percent of BrdU labeled cells in both the APC and the HPT rises and falls, with peaks on days 2, 5, and 7 in the APC and on days 3, and 6 in the HPT, before dropping by day 10 (Figure 10).

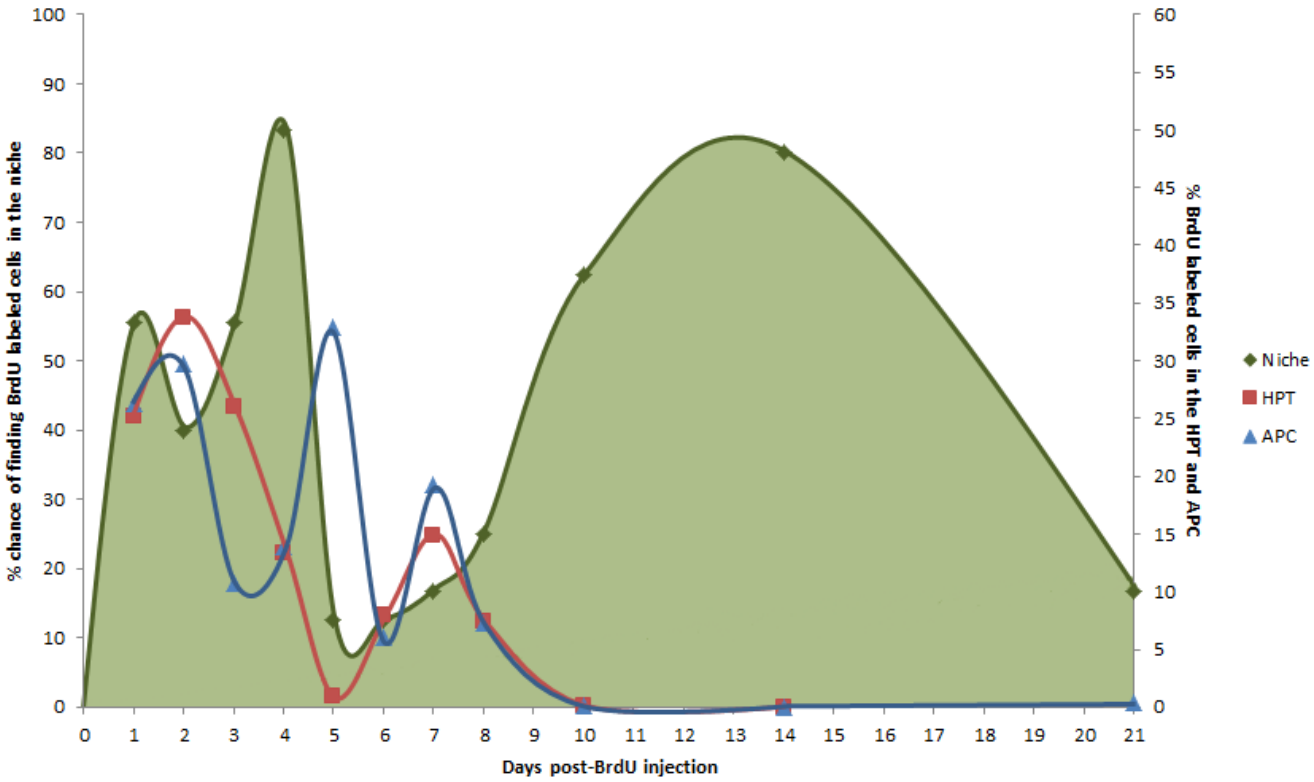


Figure 10: Following a BrdU injection on day 0 and a r-AST1 injection on day 1 the percent of BrdU-labeled cells in the immune tissues (APC and HPT) fluctuates on days 1-7 before dropping on day 8, prior to the peak in BrdU labeling in the niche on day 14. Brains, APC and HPT were dissected on days 1-8, 10, 14 and 21. The % chance of finding BrdU labeled cells in the niche as well as the % of cells that were BrdU-labeled in the HPT and APC was determined for each time point. BrdU-labeled cells are found in the niche on all days with peaks on days 1, 4 and 14.

In order to determine the effect of r-AST1 injection on the APC and HPT, the BrdU labeling patterns in each tissue for each of the three protocols were plotted against one another as shown in Figures 11 and 12.

Following simultaneous BrdU and r-AST1 injection and delayed injection of r-AST 1, there is a significant drop in BrdU-labeled cells on day 1 in the APC as compared to the BrdU only protocol (ANOVA, F=8.2446, df=2,11, P=0.0065; Tukey HSD test, BrdU

only/Simultaneous r-AST1 p=0.0103, BrdU only/r-AST1 delay p=0.0490, Simultaneous r-AST1/r-AST1 delay p=0.3808). In addition, whereas the percent of labeled cells in the APC in the BrdU only and simultaneous injection protocols decreases and does not rise again, there are a series of peaks and troughs in the percent of BrdU-labeled cells following the initial decrease of labeling in the 24 hour delay r-AST1 protocol (Figure 11).

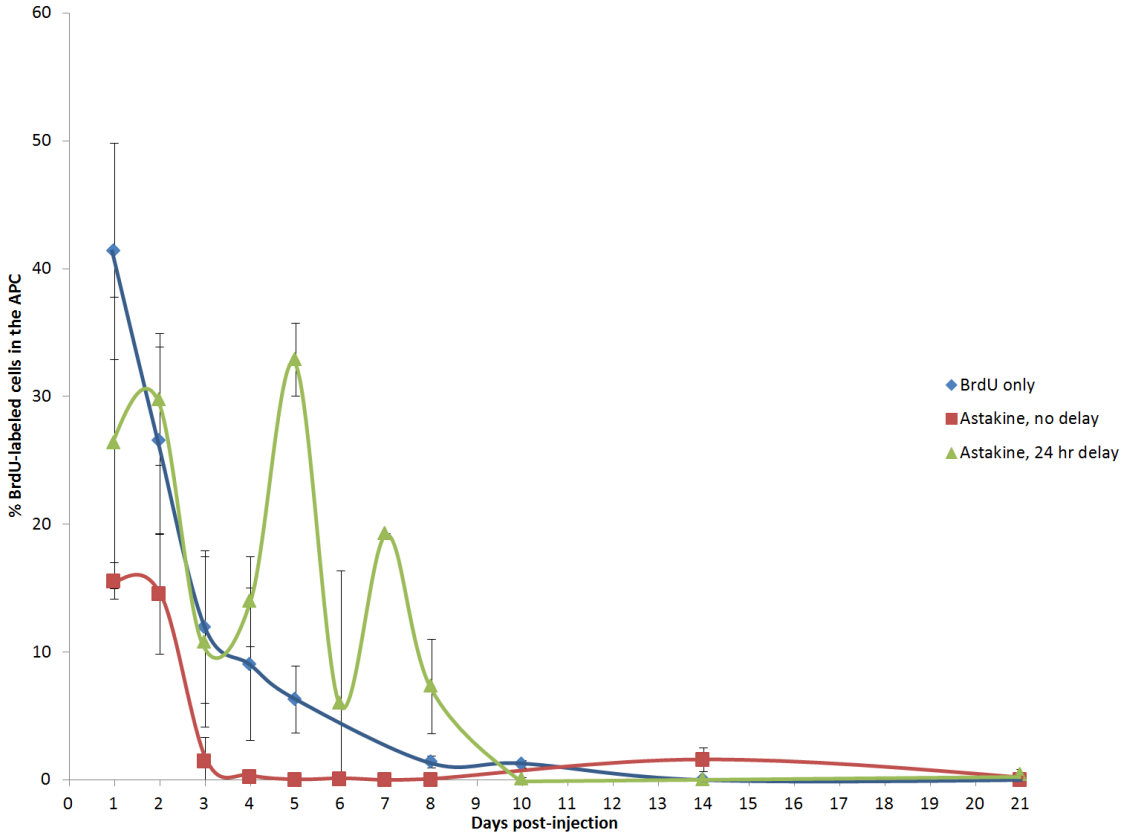


Figure 11: BrdU labeling patterns in the APC across all three experimental protocols. Following either an injection of BrdU on day 0, a simultaneous injection of BrdU and r-AST 1 on day 0, or an injection of BrdU on day 0 followed by an injection of r-AST 1 on day 1, APCs were dissected from *P. clarkii* on days 1-8, 10, 14, and 21. Samples were taken at four regions of each APC and averaged together as a single sample (shown as average +/- SD). There is a significantly larger percentage of cells labeled on day one in the BrdU only protocol than in both the BrdU only and the simultaneous BrdU an r-AST 1 injection protocol (ANOVA, F=8.2446, df=2,11, P=0.0065; Tukey HSD test, BrdU only/Simultaneous r-AST1 p=0.0103, BrdU only/r-AST1 delay p=0.0490, Simultaneous r-AST1/r-AST1 delay p=0.3808) For the BrdU only protocol: day1n=8, d2n=8, d3n=7, d4n=6, d5n=6, d6n=0, d7n=0, d8n=3, d10n=4, d14n=4, d21n=4. For the simultaneous injection protocol: d1n=2, d2n=3, d3n=4, d4n=4, d5n=4, d6n=2, d7n=3, d10n=0, d14n=1, d21n=4. For the delay protocol: d1n=4, d2n=4, d3n=0, d4n=3, d5n=2, d6n=3, d7n=1, d8n=3, d10=3, d14n=1, d21n=2.

In the HPT the percent of BrdU-labeled cells on day one does not differ significantly across all experiments (Figure 12) (ANOVA, F=0.2338, df=2,8, P=0.7967; Tukey HSD test, BrdU only/Simultaneous r-AST1 p=0.7877, BrdU only/r-AST1 delay p=0.9265, Simultaneous r-AST1/r-AST1 delay p=0.9867) In addition, while the BrdU-labeling in the HPT in the BrdU-only protocol is stable over the first three days before dropping, there is a small peak in labeling on day 2 in the simultaneous injection protocol and in the delay injection protocol. In addition, whereas the percent of BrdU labeling decreases and does not rise again in the BrdU only and simultaneous injection protocols, the labeling peaks again on day 6 in the 24 hour delay protocol.

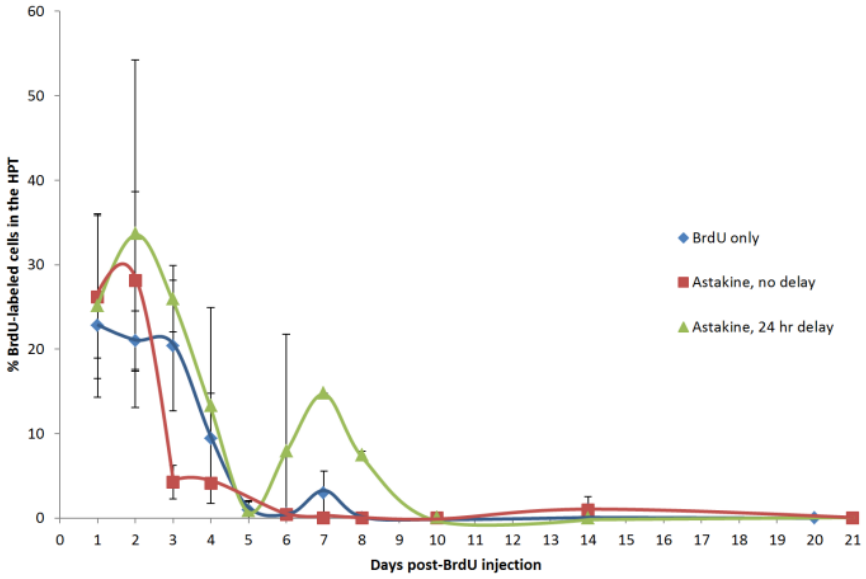


Figure 12: BrdU labeling patterns in the HPT across all three experimental protocols. Following either an injection of BrdU on day 0, a simultaneous injection of BrdU and r-AST 1 on day 0, or an injection of BrdU on day 0 followed by an injection of r-AST 1 on day 1, APCs were dissected from *P. clarkii* on days 1-8, 10, 14, and 21. Samples were taken at two regions of each HPT and averaged together as a single sample (shown as average +/- SD). For the BrdU only protocol: day1n=5, d2n=4, d3n=3, d4n=5, d5n=4, d6n=5, d7n=6, d8n=2, d10n=2, d14n=3, d20n=4. For the simultaneous injection protocol: day (d) d1n=4, d2n=4, d3n=4, d4n=2, d5n=3, d6n=3, d7n=5, d8n=3, d10n=2, d14n=2, d21n=5. For the delay protocol: d1n=2, d2n=5, d3n=1, d4n=3, d5n=2, d6n=3, d7n=1, d8n=2, d10n=3, d14n=1, d21n=0.

Discussion

The results of three experimental protocols are presented in this thesis: injection of BrdU at day 0, injection of BrdU and r-AST1 simultaneously at day 0, and injection of BrdU on day 0 followed by injection of r-AST1 on day 1. The effects of these three protocols on BrdU-labeled cells in the niche, APC and HPT were observed at standardized time points over a 21 day period. The resulting data show that manipulating the innate immune system of *P. clarkii* alters the timeline by which BrdU-labeled neuronal precursors are present in the neurogenic niche.

When *P. clarkii* were injected only with BrdU on day 0, BrdU-labeled cells were present in the niche on days 1-4 and 8-14 post-injection (Figure 7A). The observed labeling on days 1-4 was expected for a combination of reasons: (1) there is a constant population of dividing cells in the neurogenic niche that would label with BrdU; (2) the cell cycle time in the niche is approximately two days and the clearing time for BrdU in the niche is also approximately two days (Benton et al., 2011). In other words, there is a two day window during which the levels of BrdU are sufficient in the niche for a cell going through S-phase to incorporate the label at a detectable concentration. But, since it takes 2 days for the last labeled cells to divide and exit the niche, there is approximately a four day window during which cells intrinsic to the niche at the time of injection could have incorporated BrdU. As expected and as shown in Figure 7A, no BrdU-labeled cells were found in the niche by 5 days post-injection.

This experiment, however, showed that BrdU-labeled cells are again present in the niche on days 8-14 following BrdU injection. However, due to the clearing time of BrdU and the cell cycle time of cells in the niche, the cells composing this late peak (peak B in Figure 7A) could not have been labeled in the niche. Instead, these cells must have been BrdU-labeled in their source tissue, extrinsic to the niche and migrated into the niche. These data thus confirm an

extrinsic source of neuronal precursors and suggest that, since labeled cells did not reappear in the niche until day eight, it takes approximately eight days for cells to be released from their source tissue, migrate, mature and arrive in the niche.

There is strong *in vitro* and *in vivo* evidence that this extrinsic source of neuronal precursors in crayfish is the innate immune system. *In vitro* evidence shows that hemocytes (blood cells) and no other cell type are attracted to the niche in culture (Benton, Zhang et al. 2011). Additionally, *in vivo* evidence shows that the numbers of cells in the niche are tightly correlated with the total number of circulating hemocytes, as evidenced by the decrease in both cell populations following partial HPT ablation, and the subsequent rescue of both cell populations following r-AST1 injection (Benton, Kery et al. 2014). Most importantly, EdU-labeled hemocytes taken from a donor animal and injected into an unlabeled recipient animal generate labeled cells in clusters 9 and 10 that express tissue appropriate neuronal markers by seven weeks after transfer (Benton, Kery et al. 2014). Thus, the two r-AST1 injection experiments in this thesis tested whether or not manipulation of the innate immune system affects the appearance of BrdU-labeled neuronal precursors in the niche. If the source tissue is, in fact, the innate immune system, injection of r-AST1, an immune system modulator known to cause the release of hemocytes (specifically semi-granular cells [Noonin, Lin et al. 2012; Soderhall 2013]) into circulation 12 hours after injection (Benton, Kery et al. 2014) should cause BrdU-labeled cells that were labeled in the immune tissues to arrive at the niche faster than they would have in the absence of the r-AST1 injection.

As shown in Figure 7B, following a simultaneous injection of BrdU and r-AST1 on day 0, BrdU-labeled cells are present in the niche on all sampling days, including on days 5-7 when no cells were previously found. Peak E, with a 50% chance of finding BrdU-labeled cells in the

niche on day 7, a day on which no labeled cells were observed in the BrdU-only protocol, best shows this effect. As previously stated, any BrdU-labeled cells found in the niche after day 4, could not have been labeled in the niche and must have migrated to the niche after being labeled elsewhere ---- in their source tissue. Thus, the cells observed on days 5-7, a time previously devoid of cells, must have been labeled in the source tissue prior to arrival in the niche. This supports the hypothesis that the BrdU-labeled cells appearing in the niche after day 4 are coming from immune system tissues as the only difference between the two protocols was the injection of r-AST1, an immune system modulator. These results also indicate that while cells normally take about eight days to be released from the source tissue, mature, and migrate into the niche, with the r-AST 1 injection, this circulation and maturation process is quicker.

In addition to the new peak at day 7 (peak E, Figure 7B) in the simultaneous BrdU and r-AST1 injection protocol there is also a new peak on day 4 (peak D). While there was a 10% chance of finding labeled cells in the niche on day 4 in the BrdU only protocol, in this protocol there is a 50% chance. Given that r-AST1 is known to cause the differentiation and release of cells from immune tissue and into circulation 12 hours after injection, this new peak, along with the 17% and 13% chance of finding labeled cells on days 5 and 6, days where there was no labeling in the BrdU only protocol, could indicate that labeled cells from the immune tissue begin to arrive in the niche as early as 4 days post r-AST1 injection, with the majority of labeled cells ejected from the immune system by r-AST1 arriving on day 7 (peak E). This would indicate that instead of taking about eight days to circulate and mature, as seen in the BrdU-only protocol, labeled cells from the source tissue may take as little as four days to appear in the niche, but that most take seven days.

Alternately, the r-AST1 injection may have caused proliferation or self-renewing divisions of the intrinsic niche cells, thus causing the rise in both the percent chance of finding BrdU-labeled cells in the niche and in the average number of BrdU-labeled cells in the niche seen on day 4 (peak D). As shown by Benton et al. (2014), the numbers of BrdU-labeled cells in the niche rises significantly two days after injection r-AST1 injection. In the BrdU only protocol, peak A, showing the intrinsically labeled cells, lasts from day 1 to day 4. Following the r-AST1 injection in the second protocol, this peak is still present, but instead it is composed of two local maxima (peaks C and D) and an elevated level even on day 5. Peak D and the small number of cells seen on day 5 may therefore represent a division in the labeled cells that were present in the niche on days 1-3. Then, the first cells arriving from the immune system would be those seen in peak E on day 7. Even though a rise in labeled niche cells is seen on day 2 in the work of Benton et al. (2014), these data are not inconsistent with the rise seen here on day 4 (peak D) given differences in animal size between the experiments.

Another comparison of Figures 7A and B and Figure 7D shows a stark difference at day 8 post-injection where in the BrdU-only protocol there was a 37.5% chance of finding labeled cells and in the simultaneous BrdU and r-AST1 protocol there was only a 10% chance. The BrdU-labeled cells observed on day 8 in the BrdU-only protocol were the first cells to make it from the source tissue to the niche, meaning that they were likely completing S phase during the BrdU injection and were thus able to incorporate the marker and were likely also the most mature dividing cells. Thus, they would have been the first to be released from the immune system following BrdU injection and the first to reach the niche. Recall that r-AST1 causes the release of cells from the immune system tissue 12 hours after injection (Benton, Kery et al. 2014). Thus, it is logical that the cells released at the 12 hour time point were those most mature cells that

without r-AST1 would have been the cohort that first arrived in the niche on day 8. The peak on day 7 and the trough on day 8 then in the simultaneous injection experiment is likely due to the fact that the r-AST1 injection may have specifically affected that set of most mature cells that would have been most ready for release, causing them to be released and to appear in the niche beginning on day 4, and peaking on day 7. The trough then at day 8 is likely due to the fact that the r-AST1 depleted the cells in the immune system that were mature enough to be released into circulation and that would normally have taken 8 days to arrive in the niche. We hypothesize that the peak on day 10 likely represents the next cohort of cells that matured enough in the immune system to be released, following the cohort whose release was expedited by the r-AST1. In other words, the trough at day 8 is likely due to a depletion of the immune system's most mature cells caused by the r-AST 1 injection, a decrease in mature cells that took one to two days to restore before the cohort arriving in the niche at day 10 were mature enough to be released from the immune system tissue.

Given these niche timeline experiments and previous *in vitro* and *in vivo* experiments showing that the extrinsic source of neuronal precursors is the innate immune system, the percentage of cells labeled in the two major innate immune system tissues, the APC and the HPT, was determined for each time point studied. In order for the labeled cells that appear in the niche after day 4 to have come from the immune system, we would have to see a decrease in the number of labeled cells in the immune system prior to the re-appearance of labeled cells in the niche.

As shown in Figures 8 and 9, the numbers of BrdU-labeled cells in both the APC and the HPT decrease overall with time to near negligible amounts by days 4-5 in both the BrdU only and the simultaneous BrdU and r-AST1 injection protocols. This suggests that cells that divided

in the APC and HPT before the clearing time of BrdU incorporated the label and then were released from the tissues and into circulation. Figure 8 also shows that as the numbers of BrdU-labeled cells in the immune tissues drop, the numbers of EdU-labeled cells in circulation increase, showing that the labeled cells are in fact released into the circulation, with their BrdU-label.

As shown in Figure 8, while the overall trend is a decrease of BrdU-labeled cells in both tissues over time in the BrdU only experiment, the labeling decreases exponentially in the APC while it remains constant for the first three days before dropping near exponentially in the HPT. There are three possibilities for this plateau in labeling in the HPT. First, the cells from the HPT may be slower to mature and release after they pick up the BrdU-label, perhaps sitting in the HPT for three days after incorporating the label before release. Second, that the cell cycle time in the HPT may be equal to the release rate, causing release to be unnoticeable. And third, a hypothesis has been proposed that since the APC and HPT are connected, cells may migrate from the APC to the HPT, meaning that the release of cells from the HPT might not be noticeable on days 1-3 due to the fact that the tissue is being replenished by labeled cells from the APC.

Comparison of niche labeling patterns in the BrdU-only and simultaneous BrdU/r-AST1 injection shows that modulating the immune system clearly has an effect on the timeline by which BrdU-labeled cells from the source tissue appear in the niche. To further confirm this finding and to try to better determine which observed peaks were due specifically to the effects of r-AST1, a third protocol was devised in which BrdU was injected on day 0 and r-AST1 was injected on day 1. If peak E (day 7) and peak D (day 4) were due to r-AST1's effects, we

expected that by delaying the r-AST1 injection by 24 hours, these peaks would shift 24 hours to the right.

As shown in Figure 7C, using this protocol, BrdU-labeled cells were again observed at all tested time points, this time with peaks at days 1, 4, and 14. Notably, there is a trough in the percent chance of finding BrdU-labeled cells in the niche on day 2 and a stark increase of finding BrdU-labeled cells in the niche on day 4 (83.33%, up from 10% in the BrdU only protocol and 50% in the simultaneous injection protocol (peak H; Figure 7D). As above, one possible explanation for this peak is the expedited release and early arrival of cells that were labeled in the source tissue, caused by the r-AST1 injection. However, again, if the effects of r-AST1 are strictly linear, peak D on day 4 in the simultaneous injection protocol should appear on day 5 in the 24 hour delay protocol, but instead Figure 7C shows a peak of increased height on day 4, and a trough on day 2 (down from day 2 in the BrdU only experiment by 11%).

More likely, however, is the alternate explanation given above, that the r-AST1 injection induces intrinsically labeled niche cells to self-renew and/or divide, thus causing peak H. Given the likelihood that the neuronal precursors in the niche derive from the immune system, it is logical that r-AST1, an immune system modulator, would also have an effect on dividing niche cells. Of note, peak H is 33.33% higher than peak D, though I am here proposing that they are both caused by a secondary effect of r-AST1 that stimulates division of intrinsic niche cells.

As a consequence of the delay between BrdU and r-AST1 injection in this protocol, the crayfish were put through the insult of two injections, whereas they only received one injection in the other two protocols. Evidence shows that a saline injection can temporarily increase the total hemocyte count (Noonin et al. 2012). Thus, the second injection on day 1 could have caused an immune response that, similar to r-AST1 itself, caused a release of cells into

circulation. Future tests will be needed to determine whether there is an additive effect of the immune system response to an injection and to r-AST1. An additive effect between the increased total hemocyte count and the hemocyte release may have caused the increase from peak D to peak H under either hypothesis. 1) This may have caused more cells to arrive faster at the niche or 2) this may have induced a greater proliferation of the intrinsic niche cells. Given this discussion, it would be beneficial to re-do the BrdU only and simultaneous injection protocols with a sham injection of saline on day 1 to control for the non-specific effects of the injection on the immune system and total hemocyte count.

Perhaps the most noticeable change between the simultaneous and delay injection protocols is the absence of the day 7 peak (peak E) that was likely caused by the day 0 r-AST1 injection in the simultaneous injection protocol. Also noticeable is that peak I, between days 10 and 21, is taller and wider than peak B from the BrdU only protocol. Since both peak B and peak I are past the four day window during which BrdU-labeled cells intrinsic to the niche could have been labeled in the niche, cells in both peak B and I were labeled in the source tissue and then migrated to the niche. There is an increase in the percent chance of finding BrdU labeled cells in the niche from 40% to 62.5% on day 10 and from 17 % to 80% on day 14 between the BrdU only protocol and the BrdU, 24 hour delay r-AST1 protocol. If the effect of r-AST1 were strictly linear, it would have been expected that peak E on day 7 of the simultaneous injection protocol would have shifted to day 8 in the delay injection protocol, causing the graph to look similar to that in the BrdU only protocol, perhaps with an increased chance of finding BrdU-labeled cells on day 8 if the r-AST1 caused more cells than those that would normally be in the cohort of cells that picked up the labeling first.

The fact that multiple injections may have stressed the animal and caused an immune response in addition to that caused by r-AST1 may explain why peak I does not appear until day 10 when it would have been expected at day 8. Looking at Figures 8, 9, 11 and 12 it is clear there is a stark difference in the BrdU-labeling patterns in the APC and HPT between the two single-injection protocols and the double- injection protocol. While overall the labeling in both tissues decreases with time and has decreased to near negligible amounts by day 3 (simultaneous protocol) or day 4 (BrdU only protocol) with little fluctuation after the start of the decline, the percent of BrdU-labeled cells in the delayed r-AST1 injection protocol fluctuates with a second major peak in both HPT and APC before beginning a steep decline on day **8**. These fluctuations suggest that there are two important variables in the delayed r-AST1 injection protocol: the introduction of r-AST1, and the two injections. It is hypothesized that the effects of the injection and the r-AST1 are additive. In other words, the initial injection of BrdU on day 0 may have "primed" the immune system, and the second injection of r-AST1 on day 1 may have resulted in a magnified response to the cytokine, prompting divisions of cells in the APC/HPT and resulting in the secondary labeling peaks in these tissues.

Given that the BrdU-only protocol demonstrated that it takes eight days for cells that are released from the immune system to arrive in the niche, the second major peak in APC/HPT labeling explains why peak I has its maximum at day 14. As shown in figure 10, the second major peak in APC/HPT BrdU-labeling occurs at day 5 in the APC and day **7** in the HPT. The subsequent drop in labeling suggests that cells labeled in this second wave are released into the circulation in a fashion similar to those in the first wave. Thus, it would be expected that these cells would arrive in the niche approximately eight days after release, on day 13 or **15**. As shown in Figures 7C and 10, the maximum chance of finding BrdU-labeled cells in the niche occurs on

day 14, as expected according to this prediction. Peak I is then likely composed of cells migrating to the niche following the release of cells from the first and second labeling maxima (due to the possible additive effects of the r-AST1 and second injection), explaining why peak I is both wider and higher than peaks B and F.

It is also of interest to determine the pathway by which cells are released from the immune system prior to migrating to the niche. Looking at a comparison of the APC labeling patterns across the three protocols, Figure 11 shows a significant difference between the percent of labeled cells in the APC on day 1 between the BrdU only protocol and both the simultaneous and delayed r-AST 1 injection protocols. This is contrary to expectation given that on day 1 animals in the BrdU only protocol and in the delay protocol had received the same treatment. However, there is a very large standard deviation in data from day one of the delay experiment and a relatively small sample size, indicating that if there were a larger sample size, the percent of labeling should not have differed from that of the BrdU only protocol on day one. Since r-AST1 causes the release of cells from the immune tissue 12 hours after injection the fact that the percent of labeling on day one in the APC of the simultaneous injection protocol was significantly lower than that of the BrdU only protocol (and theoretically the delay protocol) indicates that the injection of r-AST1 causes the release of cells specifically from the APC 12 hours injection.

As shown in Figure 12, the percent of labeling in the HPT on day 1 does not change drastically between the three protocols, again indicating that the release 12 hours after r-AST1 injection is from the APC and not from the HPT. Additionally, there is a peak at day 2 in both the simultaneous injection experiment and in the delay experiment that was not present in the BrdU only protocol. This lends credit to the hypothesis that r-AST1 may not only cause cells to

be released from the APC into circulation, but that cells released from the APC may also migrate into the HPT prior to release. This migration of cells from the APC to the HPT following r-AST1 injection is even more likely given the fact that the peak in the simultaneous protocol is one day before the peak in the delay protocol and the r-AST1 injection in the simultaneous protocol was one day before that in the delay protocol. Alternatively, r-AST1 may promote the division of HPT and APC cells, resulting in the increases in observed counts.

In addition, Figure 12 shows that the initial drop in BrdU labeling in the HPT occurs on day 4 in the BrdU only protocol, day 3 in the simultaneous injection protocol, and day 4 in the delay protocol. The shift of release of cells to one day earlier in the simultaneous injection protocol than in the BrdU only protocol and the fact that the percent of labeled cells was not altered on day 1 as it was in the APC, suggests that the immediate effect of r-AST1 on the increase in total hemocyte count 12 hours after injection occurs because of release of cells from the APC. In addition, there may be a downstream effect in the HPT where release of cells does not occur until day 3 after r-AST1 injection, again giving credence to the possibility that cells are move from the APC to the HPT in addition to being released directly into circulation from the APC.

Conclusions

Overall, the three experimental protocols presented in the present paper 1) confirm definitively that neuronal precursors enter the niche from an extrinsic source; 2) that altering the timeline by which hemocytes are released from immune system tissues alters the timeline by which BrdU-labeled neuronal precursors appear in the niche; 3) that BrdU-labeled cells are released from both the APC and HPT and that these tissues are both potential sources of neuronal precursors; and 4) that r-AST1 causes release of cells from the APC by day 1 after injection

whereas it only alters cell release from the HPT approximately 3 days after injection. Collectively, these findings support those from Benton et al. (2011) and Benton et al. (2014) that the innate immune system is the source of neuronal precursors in the crayfish *P. clarkii* and show that these cells enter blood circulation prior to their migration into the niche.

Future studies. Further studies should be done to determine whether r-AST1 also stimulates division of the intrinsic niche cells. Planned experiments will place a desheathed brain in a dish with r-AST 1, to determine whether the cytokine alone, in the absence of hemocytes will cause an increase in the number of cells in the niche via direct effects on the niche cells.

In addition, experiments should be done to better define the roles of the APC and HPT in neuronal precursor production. This could be done by dissociating the APC and HPT and placing their cells into separate cell cultures with brains and their associated niches. The cultures would then be observed for cell attraction to the niche. However this would be inconclusive in determining the origin of the precursors if they originate in the APC, but are released from the APC both directly and by way of the HPT as the results of the current paper suggest.

In addition, it is of interest to characterize the immune cell(s) that later becomes a neuronal precursor. This work is currently being performed in our lab using Percoll gradients which allow for the separation of hemocytes by type. Each class of hemocyte (if they are labeled prior to separation) can then be injected back into a crayfish and the niche examined for the presence of labeled cells at intervals after the transfer, to identify the specific cell type that transforms into a neuronal precursor.

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